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PRINCIPAL INVESTIGATOR: Kounosuke Watabe, Ph.D.

CONTRACTING ORGANIZATION: Southern Illinois University
Springfield, Illinois 62794-9616

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Kounosuke Watabe, Ph.D.

E-mail: kwatabe@siumed.edu

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14. ABSTRACT

The proposed research aims at elucidating the function of the tumor metastasis suppressor gene, Drg-1, in the hope that we can define a specific target for novel and effective therapies to prevent metastatic disease of breast cancer. During this funding period, our major effort has been focused on Task 2b, and we identified ATF3 as a down-stream target of Drg-1 by microarray analysis. This discovery of ATF3 gene has two important implications; firstly, it opened a new direction of our research for understanding the function of Drg-1 gene, and secondly, it suggest that ATF3 can be a potential therapeutic target for the treatment of metastatic disease. We also found that the expression of Drg-1 is significantly suppressed in breast tumor cells, and as we expected, the expression was inversely correlated with patient survival. Therefore, the expression of Drg-1 can serve as a good prognostic marker. We hope that we can clarify the relationship between PTEN and Drg-1 in more detail in both in vitro and in vivo in the next fiscal year.

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INTRODUCTION

Despite significant improvement in surgical techniques and chemotherapies, none of the current medical technologies "cure" the metastatic disease (1). Metastasis is a complex biological process for which there is minimal understanding at the molecular and cellular level. The proposed research in this application aims at elucidating the function of the tumor metastasis suppressor gene, Drg-1, in the hope that we can define a specific target for novel and effective therapies to prevent metastatic disease of breast cancer. We hypothesize that Drg-1 functions as a tumor metastastsis suppressor in breast cancer (Task 1). We also hypothesize that loss of tumor suppressor PTEN down-regulates Drg-1 gene which leads to metastases (Task 2). We also plan to assess Drg-1 as a diagnostic/prognostic marker to accurately predict metastatic disease. Our ultimate goal is to develop a novel therapeutic method which mimics the function of the Drg-1 gene. We believe that the knowledge gained from the proposed study will eventually be translated into clinical trials.

BODY

Task 1-a.
To examine the effect of Drg-1 on tumor metastases in nude mouse model by injecting Drg-1 expressing cells orthotopically as well as intravenously. We will also examine the expression of the metalloprotease genes at the tumor site in the animals.

We are currently constructing breast cancer cell lines (both ER+ and ER-) that over-express Drg-1 from the CMV promoter. This cloning is in good progress. Once we obtain clones, we will inject them to Nude mice to examine the effect of Drg-1 on metastases.

Task1-b.
To perform metastasis specific microarray analysis using an inducible Drg-1 expression system to understand downstream effectors of Drg-1.

In order to identify the downstream target of the Drg-1 pathway, we performed a microarray analysis using the Affymetrix human gene array U133A. For this purpose, we first established tetracycline-inducible expression of Drg-1 in a tumor cell, and expression of the Drg-1 gene was induced by treating the cells with tetracycline or solvent alone for 48 hours. The RNA was then extracted from these cells, converted into cDNA and hybridized to the microarray. The results of our microarray analyses indicated that the ATF3 gene, a member of ATF/CREB transcription factor family (2, 3), was most significantly suppressed by induction of the Drg-1 gene.

In order to examine the effect of Drg-1 on endogenous ATF3 expression in various tumor cells, the Drg-1 expression plasmid (pcDNA3/Drg-1) or the empty pcDNA3 vector was transiently transfected into the breast cancer (MCF-7 and MDA-435) as well as prostate (PC3 and ALVA) cell lines and the level of ATF3 protein was examined by Western blot. As shown in Fig. 1A, Drg-1 attenuated the ATF3 expression in a dose-dependent manner in all these cell lines, while the empty vector did not have any notable effect. In a complementary approach, we introduced Drg-1 siRNA or GFP siRNA in the cancer cells, and as shown in Fig. 1B, the Drg-1 siRNA specifically abrogated the expression of the Drg-1 gene which led to concomitant up-regulation

of the ATF3 expression in these cells. These data strongly suggest that Drg-1 plays a crucial role the in regulation of the ATF*3* gene, and down regulation of Drg-1 in tumor cells results in augmentation of ATF3 expression. To further examine whether down-regulation of ATF3 expression by Drg-1 is mediated at the transcriptional level, tumor cells were co-transfected with Drg-1 expression vector (pcDNA3/Drg-1) or an empty vector (pcDNA3) and ATF3-CAT reporter plasmid, and the CAT reporter assay was performed. As shown in Fig. 1C, we found that the ATF3-CAT reporter activity was significantly attenuated by Drg-1, thereby strongly suggesting that Drg-1 negatively controls the expression of the ATF3 gene at the transcriptional level. We are currently testing whether the ATF3 gene indeed enhances the tumor metastases in a mouse model.

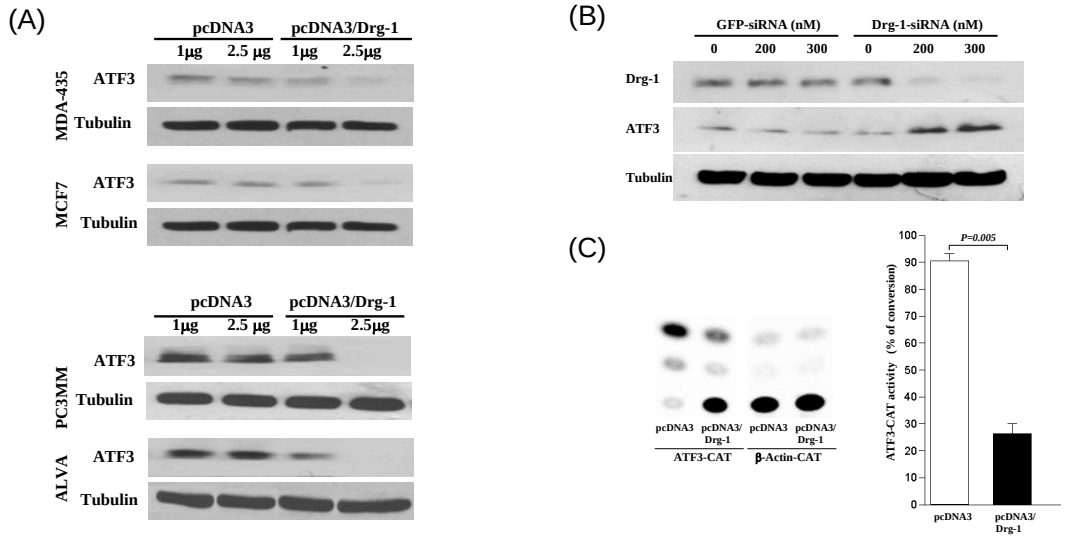

Fig.1. Drg-1 down-regulates ATF3 expression.
(A), Empty vector pcDNA3 or Drg-1 expression vector, pcDNA3/Drg-1, at the indicated amounts, was transfected into the breast cancer cell lines (MDA-435 and MCF7) and prostate cancer cell lines (PC3MM and ALVA). Forty-eight hour post-transfection, cells were lysed and Western blot was performed using antibodies against ATF3 and Tubulin. (B), siRNA for Drg-1 or GFP was synthesized and various amounts of the siRNA, as indicated, were transfected into PC3MM cells. After 72 hours, cells were lysed and the lysates were examined by Western blot with antibodies for Drg-1, ATF3 and Tubulin. (C), A CAT-reporter plasmid (ATF3-CAT) containing the ATF3 promoter region (-1850 to +34) was co-transfected with Drg-1 expression plasmid (pcDNA3/Drg-1) or empty vector (pcDNA3) into the cells. Forty eight hours later, the cells were harvested, lysed and the lysates were then assayed for the CAT activity. Acetylated chloramphenicol was resolved on TLC plate (representative run, left panel) and each spot was quantified (right panel). A reporter plasmid containing the β-actin promoter (βactin-CAT) was used as a control.

Task 2-a.
To identify the PTEN responsive region on Drg-1 promoter and the factors responsible for the activation

The reporter plasmid of Drg-1 was successfully constructed and we are currently generating systematic deletion mutants of the promoter region of the Drg-1 by using Erase-a-Base system (Promega). Once we generate a series of mutants we will co-transfect them with PTEN expression plasmid and identify the responsible regions.

Task 2-b.
To examine whether the down-regulation of Drg-1 by PTEN indeed leads to metastasis in an animal model

We have not yet pursued this Task at this point.

Task 3-a.
To examine paired samples of primary and lymph node metastases of breast cancer patients for the expression of the Drg-1 gene.

We have so far accumulated over 15 "matched" breast cancer specimens. Our IHC analysis indicates that the expression of Drg-1 is strongly down-regulated in the metastatic lesions compared to primary tumors as we expected. However, the number of samples is still too low to conduct statistical analysis. We expect to accumulate more than 50 such samples by the end of the 3rd year.

Task 3-b.
The relationship between the expression of the Drg-1 gene and recurrence of the disease will be examined retrospectively in patients over a 10 year period

In order to evaluate the prognostic value of the Drg-1 gene, univariate survival analysis was performed on 85 patients for a 5 years period. As shown in Fig.2, patients with Drg-1 positive expression had significantly more favorable prognosis than those with reduced expression of the gene (P=0.002, log rank test). Thus, the reduced expression of Drg-1 can be a strong predicator of lymph node and bone metastasis and, in turn, of survival. Therefore, these data underscores the clinical relevance of this gene in advancement of breast cancer. We are currently collecting more samples to asses the patient outcome for a period of 10 years.

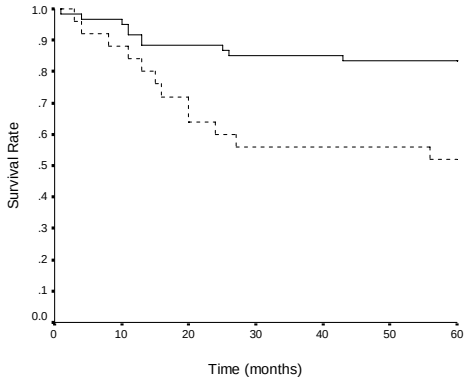

Fig.2. Drg-1 expression is correlated with survival rate in breast cancer. Disease-free survival rate over a period of 5 years was analyzed in 85 patients in relation to Drg-1 expression. Solid line and dotted line indicate Drg-1 positive patients and patients with reduced expression of Drg-1, respectively. P value was determined by log rank test.

Task 3-c.
To evaluate the status of PTEN, and Drg-1 expression and their relation to survival of breast cancer patients

We performed an immunohistochemical analysis on an archive of limited number of breast cancer tissue samples. The results showed that Drg-1 was expressed strongly in the epithelial

cells of normal ducts and glands in both prostate and breast tissue sections, while the poorly differentiated tumor cells in the same specimen had significantly reduced level of Drg-1. Similarly, PTEN was also found to be highly expressed in the epithelial cells of normal ducts and glands, where the protein was detected mostly in the cytoplasm. Importantly, as shown in two representative fields in Fig.3, almost identical staining pattern was obtained when the same field was examined for PTEN and Drg-1 expression. We are currently continuing immunohistochemical staining for more number of samples so that we can conduct statistical analysis.

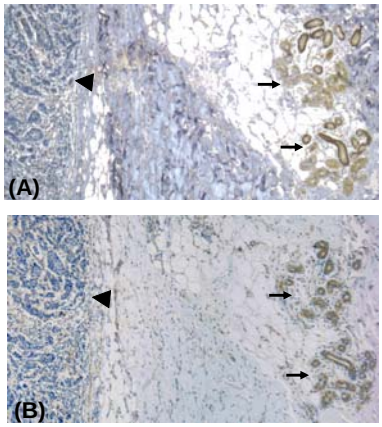

Fig.3. Immunohistochemical analysis of Drg-1 with respect to PTEN and other clinico-pathological parameters in human breast cancer. **(A)** Immunohistochemistry for Drg-1 and PTEN was performed on paraffin tissue sections. A representative field from a breast cancer specimen immunostained with Drg-1 (A) and PTEN (B) antibodies.

KEY RESEARCH ACCOMPLISHMENTS

1. We have identified the ATF3 gene as a down-stream target of Drg-1 by microarray analysis.
2. We have proven that ATF3 is indeed the direct target of Drg-1 in vitro by using over-expression as well as siRNA knockdown of the Drg-1 gene.
3. We have examined the expression of Drg-1 in tumor tissues from breast cancer patients and found that Drg-1 is significantly down-regulated in metastatic tumors and the level of the expression is inversely correlated with 5-year survival of patients.
4. Immunohistochemical analysis of breast tumor samples indicates that there is strong positive correlation between PTEN and Drg-1 expression.

REPORTABLE OUTCOMES

Peer reviewed publications

1. Bandyopadhyay, S., Fulk R.S., Pai, SK., Gross, SC., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Saito, K., Watabe, M., Wang Y., Huggenvik, J. Pauza, ME, Iiizumi, M. and Watabe K.(2005) FAS expression inversely correlates with PTEN level in prostate cancer and an Akt inhibitor synergizes with FAS siRNA to induce apoptosis. *Oncogene,* 24, 5389

Abstract/presentation

1. Bandyopadhyay, S., Wang, Y., Pai, S.K., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Takano U., Saito, K., Commes, T., Piquemal, D., Watabe, M., and Watabe K. (2005) The metastasis suppressor gene Drg-1 downregulates the expression of ATF3 in prostate and breast carcinoma. American Association for Cancer Research. Anaheim, CA.
2. Bandyopadhyay, S., Pai, S.K., Watabe, M., Gross, S.C., Hirota, S., Hosobe, S., Tsukada T., Miura, K., Saito, K., Markwell S.J., Wang Y., Zhan R., and Watabe K. (2005) FAS expression inversely correlates with the expression of PTEN and an Akt inhibitor synergizes with FAS siRNA to induce apoptosis in prostate cancer cells. American Association for Cancer Research. Anaheim, CA.

Employment

1. Dr. Sucharita Bandyopadhyay (Postdoc) has been supported by the current grant.
2. Dr. Eiji Furuta (Postdoc) has been partly supported by the current grant.

CONCLUSIONS

During this funding period, our major effort has been focused on Task 2b. Our finding indicates that ATF3 is the direct down-stream target of Drg-1. This is a long-waited break-through in our research. We have confirmed this observation by various in vitro approaches. ATF3 is a transcription factor and was previously suggested to be involved in tumor metastases (4). We now need to understand how ATF3 promotes metastasis and how Drg-1 suppresses the ATF3 gene. We also found that the expression of Drg-1 is significantly suppressed in breast tumor cells, and as we expected, the expression was inversely correlated with patient survival. Therefore, the expression of Drg-1 can serve as a good prognostic marker. We hope that we can clarify the relationship between PTEN and Drg-1 in more detail in both in vitro and in vivo.

So what?
Our discovery of the ATF3 gene as a down-stream target of Drg-1 has important implications. Firstly, this opened a new direction of our research for understanding the function of this suppressor gene. Secondly, this finding suggests that ATF3 can be a potential therapeutic target for the treatment of metastatic disease.

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3. Chu HM, Tan Y, Kobierski LA, Balsam LB, Comb MJ. Activating transcription factor-3 stimulates 3',5'-cyclic adenosine monophosphate-dependent gene expression. Mol Endocrinol 1994;8:59-68.
4. Ishiguro T, Nagawa H. Expression of the ATF3 gene on cell lines and surgically excised specimens. Oncol Res 2000;12:181-3.